

# NT157 Inhibits Hepatocellular Carcinoma Growth and Sensitizes Cells to Sorafenib

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**Research**

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# Abstract

**Background:** Sorafenib has been recognized as the standard therapy for advanced hepatocellular carcinoma (HCC). Besides, efficacy of sorafenib was unsatisfactory and vast patients are resistant to sorafenib. Thus, molecular mechanisms underlying regulation of sorafenib resistance and seeking potential strategy to improve its efficacy have attracted much attention. As a small-molecule inhibitor of IGF-1R, NT157 has potent antitumor activity against some human cancers. However, whether NT157 has potential anti-tumor effects and its molecular mechanisms in HCC remain poorly understood.

**Methods:** We assessed the effects and explored the mechanism of NT157 and sorafenib as single agents or in combination with sorafenib in HCC cells and mouse model. Further, we further demonstrated that NT157 reversed resistance to sorafenib in HCC.

**Results:** Here, we found NT157 inhibited HCC growth and induced apoptosis *in vitro* and *in vivo*. In terms of mechanism, NT157 phosphorylated IRS-1 through ERK-MAPK signaling to be degraded by the ubiquitin-proteasome pathway, lowered p-AKT to deactivate IGF-1R signaling to inhibit proliferation and induce apoptosis. Surprisingly, we further demonstrated that NT157 acted synergistically with sorafenib to inhibit proliferation and contributed to sensitize HCC cells to sorafenib by down-regulation of p-AKT.

**Conclusions:** Overall, our findings provide a translational rationale for inhibition of IGF-1R and downstream signaling pathways by NT157 as a novel targeted therapy alone or combined with sorafenib in HCC.

## Background

Hepatocellular carcinoma (HCC) is one of the most lethal primary malignant tumors and especially almost fifty percent of new cases are in China. When diagnosed, the vast majority of patients are unresectable<sup>1</sup>. Due to limitation of therapies, the 5-year survival rate is only 3% to 11% in advanced HCC patients<sup>2</sup>. Tyrosine kinase inhibitor sorafenib has been approved to treat advanced HCC and remains first-line treatment, however, it is beneficial in only one third of the patients. Besides, acquired resistance of sorafenib often develops within 6 months and it is still the primary obstacle for long-term survival of HCC<sup>3-4</sup>.

As sorafenib targets multiple signal pathway, different mechanism might induce its resistance, including the activation of compensatory signaling cascades, alterations of the microenvironment, and cell plasticity<sup>5</sup>. Although previous studies have revealed drug resistance of sorafenib, no target has yet been translated into clinical application at present. A growing number of studies suggest that

IGF-1 and its downstream in solid tumor are responsible for chemoresistance and tumor relapse leading to disease progression and mortality. Some evidence reveals that IGF-1R and AKT are essential for tumor cells to adapt to sorafenib<sup>6</sup>. Notably, the expression and the exact functions of IGF-1R and AKT, in terms of cell surviving, are still ambiguous in sorafenib resistance of HCC. Therefore, therapeutic strategies

targeted alternative signaling pathway have enormous potential as they may circumvent, at least partially, the development of drug resistance.

NT157 is a new small-molecular inhibitor, which has been used for the treatment of various tumors.

However, there has no deep research on HCC up to now. In this present study, we found that NT157 inhibits HCC growth by targeting IGF-1R/IRS1 pathway and downstream activation of the ERK/MAPK pathway. Combining NT157 and sorafenib exhibits synergy against HCC via ERK signaling. Furthermore, NT157 can sensitize sorafenib-resistant cells by down-regulation of p-AKT. Collectively, our findings not only identify the role and mechanism of NT157 in HCC but also provide evidence the combination of NT157 and sorafenib has therapeutic potential for HCC.

## Materials And Methods

### HCC cell lines

Human HCC cell lines HepG2, SMMC-7721, Hep3B, MHCC-97L and human normal liver cell line LO2 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin-streptomycin at 37°C in 5% CO<sub>2</sub> atmosphere.

### Cell viability assay and evaluation of drug interaction

Cell viability was measured using MTT (Sigma) according to the manufacturer's protocol. HCC cells were seeded in a 96-well plate (5000 cells/well), and 20 µL MTT solution was added at the indicated time points. After 4 hours, the absorbance (450 nm) was measured. Synergy scoring was determined using the inhibition readout (calculated as 100-cell viability) on the online SynergyFinder software (<https://synergyfinder.fimm.fi>) and implementing the BLISS calculation method<sup>7</sup>.

### Establishment of sorafenib-resistant HCC cells

To develop sorafenib-resistant cells HepG2-SR, HepG2 cells were treated stepwise with increasing doses of 0.25 µM sorafenib (Selleck, Houston, TX, USA) per week for 6 to 8 months. And HepG2-SR were cultured in medium containing 5 µM sorafenib to maintain the acquired resistance.

### Animal studies

Five-week-old male BALB/c nude mice were purchased from the Shanghai Experimental Animal Center and were housed in a pathogen-free facility at the Animal Center of Xi'an Jiaotong University. The animal experiments were approved by the Animal Experiment Administration Committee of Xi'an Jiao Tong University. Humane care of animals conformed with the "Guide for the Care and Use of Laboratory Animals" criteria of the National Academy of Sciences (National Institutes of Health publication 86-23, revised 1985). 1×10<sup>6</sup> HepG2 cells were injected subcutaneously to establish the subcutaneous mouse

xenograft model. Once solid tumors reached a volume of 100 mm<sup>3</sup>, mice were treated with NT157 (100 mg/kg, intraperitoneal injection or 2 μM, intratumoral injection), and/or sorafenib (15 mg/kg, oral gavage) once every two days for 16 days. Tumor volume was calculated using the following formula: tumor volume (mm<sup>3</sup>) = 1/2 × (long diameter) × (short diameter)<sup>2</sup>. Data collection was stopped at ethical endpoints, including deterioration in health or when tumors reached ≥ 4 times the initial tumor volume at enrollment.

## Statistical analysis

All statistical analyses were carried out by GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) or SPSS software (version 18.0; SPSS Inc., Chicago, IL). For all experiments, *P* values were determined using two-tailed Student's t-test. For multiple comparisons of parametric data, the one-way ANOVA test followed by Tukey's multiple comparisons test were used. Chi-square or Fisher's exact test was used to compare categorical variables. Correlations were analyzed between CCL20, CXCL8 and AFP. The Kaplan-Meier method was used to evaluate overall survival (OS), and the log-rank test was used for survival comparisons. Results are expressed as mean ± S.D. from an appropriate number of experiments (at least three biological replicas) and statistical significance was set at \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

## Results

### NT157 inhibits HCC growth *in vivo* and *in vitro*.

In search of acquiring the potential value of IGF-1R as a therapeutic target in HCC, we analyzed

*in silico* the correlation between IGF-1R RNA expression and overall survival (OS). Using of TCGA database, IGF-1R overexpression was found to be associated with poor overall survival in HCC (Supplementary Fig. 1, Supplementary Table1).

Next, we directly detected potential therapeutic value of NT157 *in vivo* and *in vitro* experiments.

Different HCC cells were incubated with various concentrations of NT157 for various time. The data showed that NT157 significantly inhibited the proliferation of HCC cells in a dose-dependent and time-dependent manner (Fig. 1a). Colony formation assays corroborated the inhibition of proliferation by NT157 treatment (Fig. 1b). To test whether NT157 could induce apoptosis, we also measured the apoptotic ratio of HCC cells in response to NT157 treatment. Results showed that NT157 treatment resulted in marked increases in apoptosis as measured by Annexin V and caspases-3 and PARP cleavage, suggesting the activation of the apoptotic cascade (Fig. 1c-d). Furthermore, to verify the antitumor effects of NT157 against HCC *in vivo*, we conducted subcutaneous mouse xenograft model by intraperitoneal and intratumor administration, respectively. In both administration methods, NT157-treated mice exhibited significant tumor growth inhibition in sharp contrast to control groups (Fig. 1e).

Notably, HCC cell lines were sensitive to NT157, whereas normal hepatocyte LO2 was tolerant to NT157 treatment (Supplementary Fig. 2a). In both subcutaneous mouse xenograft model, NT157 had no significant effect on the body weight of tumor-bearing mice (Supplementary Fig. 2b). Furthermore, NT157 had no obvious effect on routine blood test and liver and renal function test (Supplementary Table2). Serum concentrations of Alpha-fetoprotein protein (AFP) are commonly and classically used for the evaluation of tumor burden of HCC patients. To evaluate the effect of NT157, AFP level was assessed by ELISA. Results showed NT157 reduced the AFP levels in HCC cells (Supplementary Fig. 3a), suggesting that tumor burden may be decreased after NT157 treatment. Further, to seek the efficacy biomarker of NT157, RNA-seq, qRT-PCR and flow fluorimetry were performed. And CCL20 and CXCL8 had the most noteworthy change in the NT157-treatment group (Supplementary Fig. 3b-c). In addition, the relative expression in CXCL8, CCL20, and AFP had remarkably negative correlations (Supplementary Fig. 3d), which intensively implied that CXCL8 and CCL20 may be an efficacy biomarker of NT157. Taken together, these data demonstrated that NT157 may inhibit HCC growth *in vivo* and *in vitro* effectively and safely.

### **NT157 targets IGF-1R/AKT pathway by activation of the ERK/MAPK pathway**

To determine the mechanisms of NT157, HCC cells were subjected to RNA-seq analysis to quantitatively detect expression profile changes in mRNA. A total of 3738 differentially expressed genes (DEGs) were identified (Fig. 2a). DEGs were mostly involved in cell cycle progression, proliferation, and apoptosis (Fig. 2b). And Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis implied the significant pathway, MAPK signaling pathway (Fig. 2c).

We found that NT157 induced extensive phosphorylation of IRS1 proteins in HepG2 and SMMC-7721 cells (Fig. 3a). And, with time increasing, p-IRS induced by NT157 decreased (Fig. 3b). Furthermore, to explore the mechanism of p-IRS degradation, we combined treatment with NT157 and MG132, a proteasome inhibitor, led to p-IRS1 accumulation (Fig. 3c), suggesting that NT157 induced IRS1 phosphorylation and subsequent IRS1 degradation. Surprisingly, the downstream activation of AKT was also suppressed in HepG2 cells treated with NT157 (Fig. 3d). It has been reported that shutting off IGF-1R signaling depended on the targeting of IRS1 phosphorylation and ubiquitin proteasome-dependent degradation<sup>8-9</sup>. Hence, it showed that targeting IGF-1R by NT157 depended on degrading p-IRS1 and subsequently inhibiting downstream AKT. Besides, we found that NT157 could induced ERK pathway activation (Fig. 3a). To detect the relationship between IRS1 and ERK pathway, treatment of HepG2 cells with the RAF/ERK pathway inhibitor PLX4720 showed that PLX4720 abolished NT157-induced increase in phosphorylation of IRS1 and ERK (Fig. 3e). It proved NT157-induced phosphorylation of IRS1 was dependent on the ERK pathway. Besides, combined treatment of PLX4720 abrogated the anti-proliferative effects of NT157(Fig. 3f). Thus, these results indicated that NT157 induced the activation of the ERK-MAPK pathway, leading to phosphorylation and degradation of IRS1, and in turn blocked IGF-1R/IRS1/AKT signaling to induce cell death.

### **Combining NT157 and sorafenib exhibits synergy against HCC via ERK signaling.**

A recent report indicates that ERK signaling is involved in enhancing sensitivity to the sorafenib<sup>10</sup>. As NT157 treatment results in ERK activation, we then evaluated the effect of combined treatment of NT157 and sorafenib, trying to figure out whether it is additive, synergistic or antagonistic. First, we verified that sorafenib, which has been proved to block ERK signaling pathway<sup>11</sup>, inhibited HCC cell proliferation and lowered ERK expression (Supplementary Fig. 4a-b). Then, we found that monotherapy with either sorafenib or NT157 reduced HCC cell growth, and combination of sorafenib and NT157 intriguingly exerted greater growth inhibition than either agent alone, suggesting that NT157 treatment significantly enhanced sorafenib cytotoxicity (Fig. 4a). Meanwhile, we observed a synergistic effect by NT157 and sorafenib, as demonstrated in Loewe plots and calculated Bliss combination indices (CIs)<sup>11</sup> (Fig. 4a). Importantly, although a synergistic effect was observed in HCC cell lines, no significantly cooperative lethality appeared in normal hepatocytes (Supplementary Fig. 4c). Moreover, apoptotic rate was also found to be increased as measured by flow cytometer and apoptotic related proteins in HCC cell lines. We found that compared with untreated control, NT157 or sorafenib alone induced apoptosis to some extent, while the combination induced more apoptosis (Fig. 4b). Similarly, the cleavage of caspase-3 and PARP were increased in HCC cells exposed to NT157 or sorafenib alone and were further enhanced in cells treated with the combination (Fig. 4c).

Given the strong synergy of these 2 drugs in HCC, we next investigated the possible mechanisms of combination therapy. We found that when sorafenib was administered, NT157-induced ERK activation was attenuated (Fig. 4d). Combined treatment resulted in inhibition of both ERK and AKT pathways. Importantly, a sequential treatment of NT157 and sorafenib was crucial to gain a synergistic effect. Potentiated cytotoxicity by NT157 plus sorafenib might be partially due to ERK suppression by sorafenib in HCC cells. The synergistic effect *in vitro* prompted an evaluation *in vivo*. In our xenograft model, animals were randomly treated with vehicle, NT157, sorafenib, or NT157 and sorafenib in combination. We found that both NT157 and sorafenib significantly suppressed HCC xenograft growth, while suppressed more significantly in the combination cohort compared with single agent (Fig. 4e). Meanwhile, no significant weight loss and significantly cooperative lethality of vital organs were observed compared with sorafenib alone during this treatment period (Fig. 4f, Supplementary Fig.5), indicating that the combination was well tolerated. Immunohistochemical analysis showed increased TUNEL-positive tumor cells and decreased Ki67 in the combination cohort (Fig. 4g). Overall, the combination of NT157 and sorafenib exhibited significant synergy against HCC, likely through p-ERK suppression.

### **NT157 can sensitize HCC cells to sorafenib by down-regulation of p-AKT.**

After long term exposure to sorafenib, almost all patients would emerge acquired resistance, and eventually progress<sup>3-4</sup>. To this end, we developed HCC cell line resistant to sorafenib *in vitro*, HepG2-SR. This cell line was cloned from the parental HCC cell line HepG2, and the relative resistance index was 2.10 times higher in sorafenib-resistant HepG2 cells than parental HepG2 cells (Fig. 5a). Apoptotic rate of HepG2 cells was more than 2-fold higher than HepG2-SR cells with 5 and 10  $\mu\text{mol/L}$  of sorafenib, respectively (Fig. 5b).

Surprisingly, we found a significant increase of p-AKT and its downstream p-mTOR in HepG2-SR cells, compared with the corresponding parental HepG2 cells, which are sensitive to sorafenib (Fig. 5c). The results indicated that sustained exposure to sorafenib would lead to AKT activation, conforming the previous studies<sup>6,13-15</sup>. As we found the suppression of AKT signaling in HepG2 cells treated with NT157 (Fig. 3d), which prompted us to investigate whether NT157 can reverse sorafenib-resistance of HCC.

Furthermore, we assessed the efficacy of NT157 and sorafenib, in combination and as single agents, on established sorafenib-resistant HCC cells *in vitro*. NT157 strikingly inhibited cell viability, and the inhibitive effect of combination of these 2 drugs on cell viability was more remarkable in HepG2-SR cells (Fig. 5d). Besides, we also demonstrated a combination of sorafenib and NT157 significantly increased apoptosis in HepG2-SR cells (Fig. 5e). The increased apoptosis shown by the expression of caspase-3 and PARP indicated that sorafenib-resistant cells were refractory to sorafenib-induced apoptosis through caspase-dependent and -independent ways, while NT157 sensitized resistant cells to sorafenib-induced cell death (Fig. 5f). Moreover, in HepG2-SR cells NT157 induced striking p-AKT inhibition, and combined treatment with NT157 and sorafenib attenuated p-AKT increase compared with HepG2-SR cells treated with sorafenib, a finding that suggests NT157 could sensitize HCC cells to sorafenib by inhibiting AKT signaling pathway (Fig. 5f). In summary, we found p-AKT level was increased in sorafenib-resistant HCC cells and NT157 may be a potential drug for reversing sorafenib resistance by inhibiting AKT signaling in HCC.

## Discussion

In the study, we found that NT157 inhibits HCC growth both *in vitro* and *in vivo*. NT157 phosphorylates IRS-1 through ERK-MAPK signaling to degrade by the ubiquitin-proteasome pathway, lowers p-AKT to turn off IGF-1R signaling to inhibit proliferation. Furthermore, NT157 and sorafenib provide synergistic effects to inhibit proliferation, and NT157 can sensitize cells to sorafenib by lowering p-AKT levels. Our findings not only illustrate the mechanism of NT157-mediated growth inhibition by targeting IGF-1R/IRS1/AKT signaling but also provide a novel strategy of combining NT157 and sorafenib, which may be a promising therapeutic approach in HCC (Fig. 6).

Given the observed negative correlation between IGF-1R expression and OS in patients with HCC, we focused on the possibility of targeting IGF-1R by NT157 in HCC. Our results showed that NT157 significantly inhibited HCC growth and induced apoptosis *in vitro* and *in vivo*. In terms of mechanism, we demonstrated that NT157 induced ERK-MAPK activation to phosphorylate IRS1. Then phosphorylated IRS1 underwent degradation to shut off long-lasting IGF-1R/IRS1/AKT signaling to exert effect. IGF-1R is highly homologous to insulin receptor (IR) in structure and function. It has been demonstrated that monoclonal antibodies of IGF-1R can induce compensatory activation of IR, leading to drug resistance<sup>16</sup>. As IRS1/2 mediates signaling pathway from both IGF-1R and IR, NT157 leads to the disruption of signaling downstream of both receptors induced by IGF1, IGF2 or insulin and reduce the probability of drug resistance. Taken together, these data demonstrated that NT157 was well effective by inhibiting IGF-1R/IRS/AKT signaling to prevent HCC growth.

In our study, we found ERK activation after NT157 treatment. As it has been previously shown that the higher the p-ERK level is in HCC, the more sensitive the tumor is to sorafenib, suggesting that p-ERK is a potential predictor of sensitivity to sorafenib when treating HCC<sup>10</sup>, we further explored the possibility and rationale of combination treatment with sorafenib and NT157. We demonstrated a synergistic effect between NT157 and sorafenib in HCC cell lines, and combination therapy yielded reductions in tumor growth in HCC. In addition, our findings showed that combining sorafenib and NT157 offered increased benefits against HCC likely through the mechanism of inhibiting NT157-induced ERK activation after a sequential treatment. However, the long-term effectiveness of sorafenib is still controversial because the acquired resistance has been observed. Activation of alternative pathways to counteract targeted therapies leads to sorafenib resistance. Similarly to our data, growing evidence implies an important role of IGF-1R and AKT signaling in conferring resistance in human malignancies, consisting of a study which highlighted the role of AKT in sorafenib resistance<sup>13,15</sup>. Our results showed that NT157 could negatively regulate p-AKT. Therefore, we further focused on exploring the effect of NT157 on sorafenib-resistant HCC cells. We found that sorafenib activated AKT in sorafenib-resistant HCC cells. Surprisingly, NT157 successfully decreased sorafenib dosage and sensitized cells to sorafenib with the reduction of p-AKT in sorafenib-resistant HCC cells. These data have suggested that IGF-1R/AKT pathway activation maybe a sorafenib-resistant mechanism and NT157 could serve as a novel targeted therapy combined with sorafenib in HCC.

Although targeted IGF-1R inhibition alone or in combination with other therapies has achieved antitumor responses in a wealth of different tumors, IGF-1R inhibitors are associated with fatal events and a narrow therapeutic index<sup>16-18</sup>. Given these constraints and therapeutic potential, exploring novel therapeutic strategies is warranted.

## Conclusions

Our findings provide rationale for testing NT157 and developing the combination of sorafenib in HCC. In our study, we found NT157 had good safety no matter monotherapy or combination. Therefore, it provides the possibility for clinical application in the future as its promising clinical efficacy and safety. Taken together, our findings provide a translational rationale for inhibition of IGF-1R and downstream signaling pathways by NT157 as a novel targeted therapy alone or combined with sorafenib in HCC. And NT157 may potentially act as an enhancer of sorafenib to inhibit HCC growth.

## Abbreviations

ELISA: enzyme-linked immunosorbent assay; HCC: hepatocellular carcinoma; HR: hazard ratio; IGF: insulin-like growth factor; IGF-1R: insulin like growth factor 1 receptor; IRS-1: insulin receptor substrate-1; OS: overall survival; qRT-PCR: quantitative real-time polymerase chain reaction; SD: standard deviation.

## Declarations

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## **Author's contributions**

Study concept and design: Hui Guo, Yu Wang, Si-Zhe Yu; Acquisition of data: Yu Wang, Si-Zhe Yu, Jia Hou, Shi-Rong Zhang, Wen-Yuan Li; Analysis and interpretation of data: Min Jiao, Tao Tian, Zhi-Ping Ruan, Xuan Liang, Wen-Juan Wang; Drafting of the manuscript: Yu Wang, Si-Zhe Yu; Critical revision of the manuscript for important intellectual content: Ke-Jun Nan, Chang Liu, Yi Lv, Francois X. Claret, Hui Guo.

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## **Availability of data and materials**

The datasets supporting the conclusions of this article are included within the article and its Additional files

## **Ethics approval and consent to participate**

The Ethics Committee of Xi'an Jiaotong University approved this study. The animal experiments were approved by the Animal Experiment Administration Committee of Xi'an Jiao Tong University.

## **Conflict of interest disclosure**

The authors declare that they have no conflict of interest.

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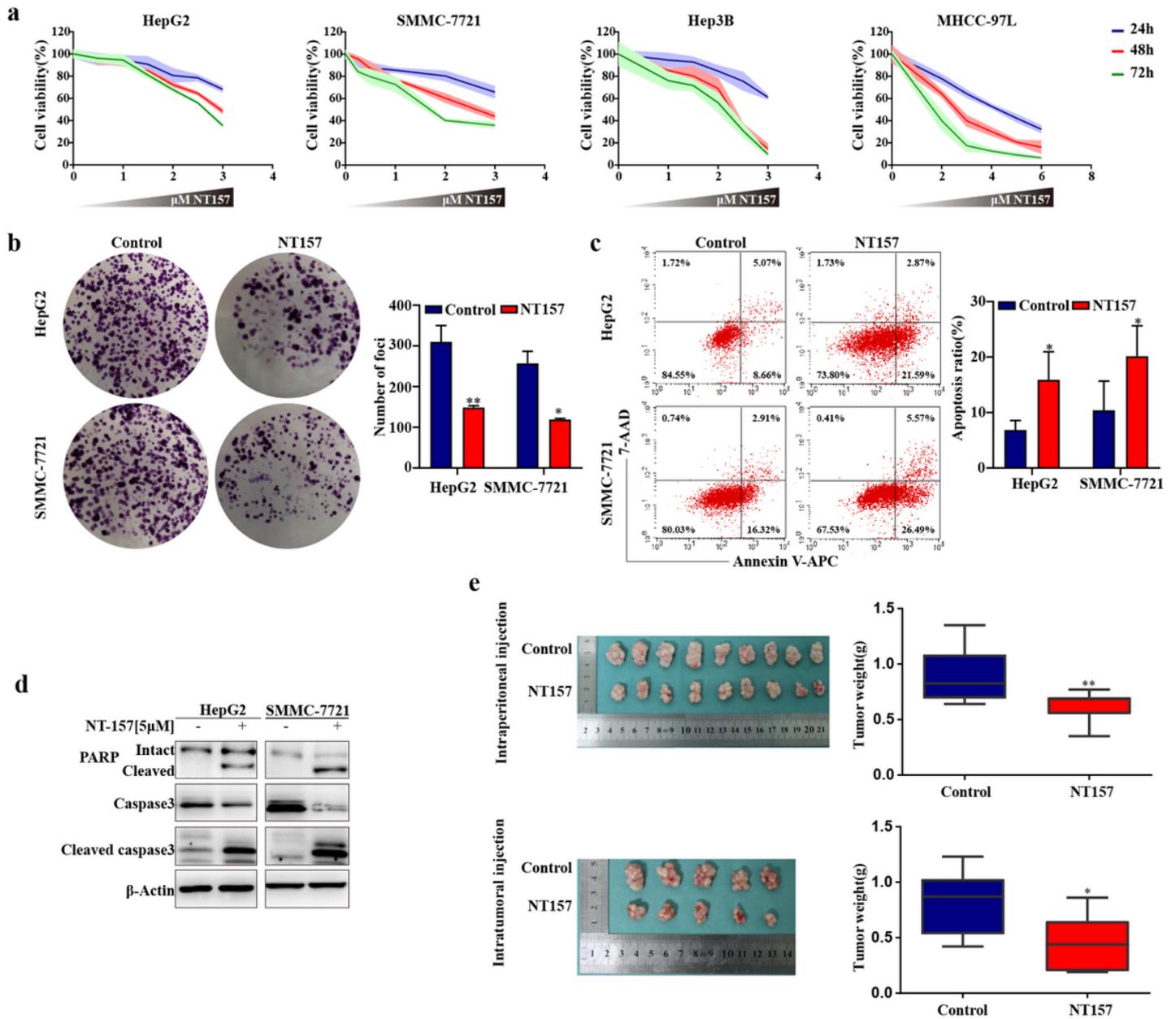
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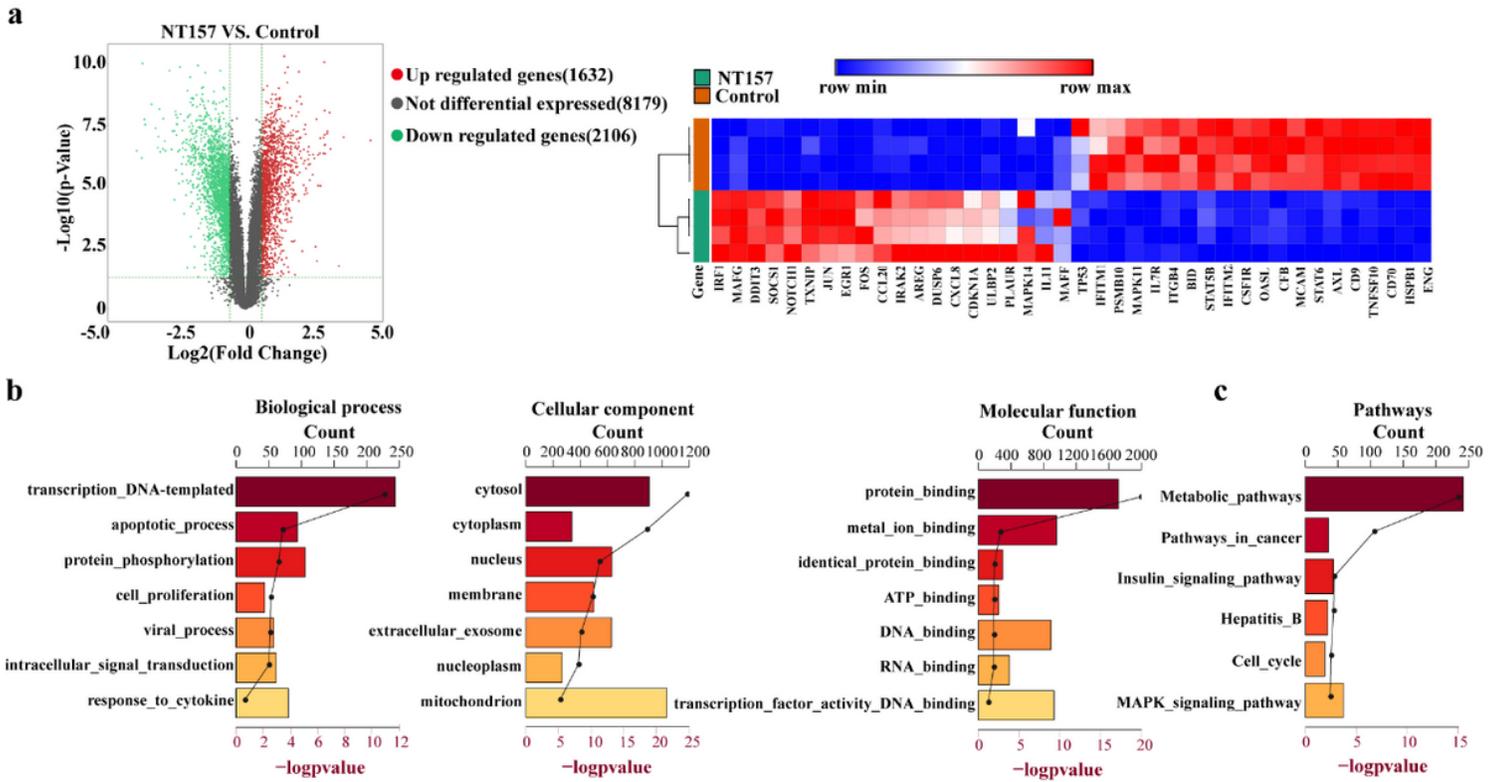
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## Figures



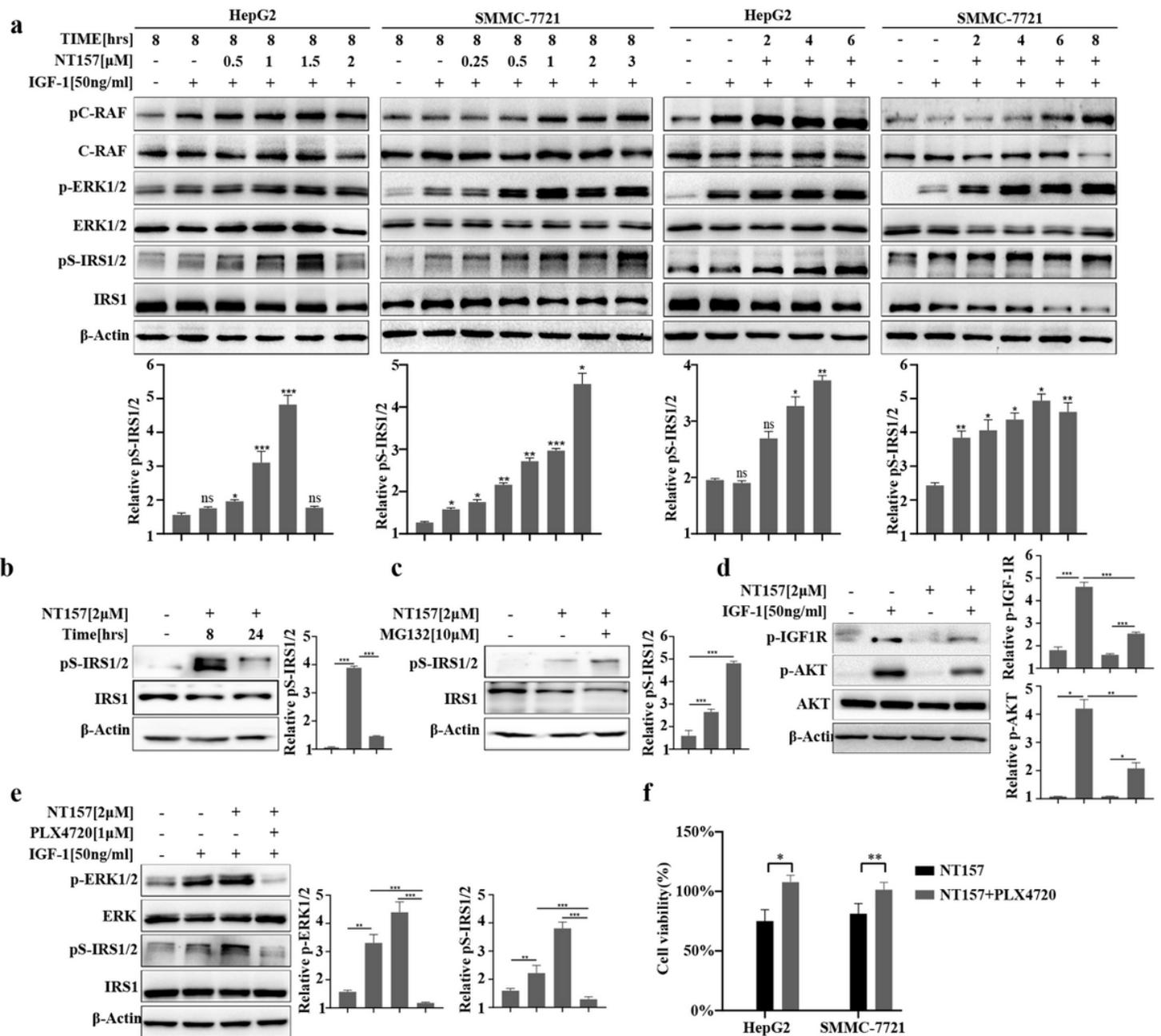
**Figure 1**

NT157 demonstrates antitumor activity in vitro and in vivo. a NT157 inhibited HCC cells proliferation. Treatment of 4 HCC lines (HepG2, SMMC-7721, Hep3B, and MHCC-97L) with different concentrations of NT157 for different times. Cell viability was measured by MTT assay. b Colony formation activity of HCC cells treated with NT157 was decreased compared with controls. c Annexin V-APC/7-AAD staining demonstrated increased apoptosis of HCC cells when treated with NT157. d Enhanced the expression of apoptosis-related proteins (cleaved PARP and caspase-3) following NT157 drug treatment. e NT157 inhibited significantly HCC tumor growth. Experiments were repeated 3 times. Error bars, mean  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .



**Figure 2**

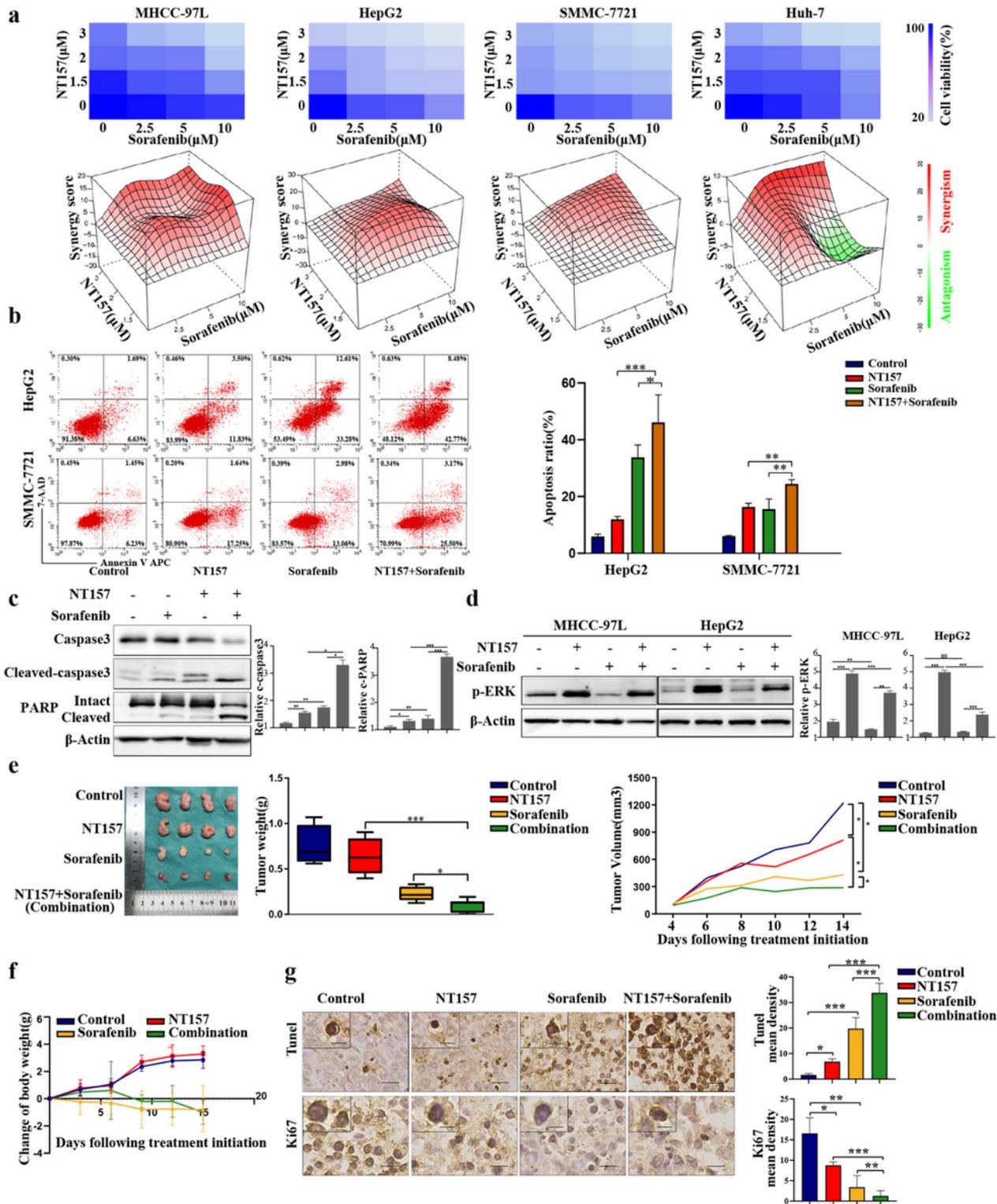
Differentially expressed genes and analysis of enrichment. a Differentially expressed genes (DEGs) and heat map of the top 40 DEGs. b Gene ontology of DEGs. c Kyoto Encyclopedia of Genes and Genomes pathway map of DEGs.



**Figure 3**

NT157 targets IGF-1R pathway by activation of the ERKMAPK pathway. a NT157 induced IRS1/2 Ser-phosphorylation and ERK-MAPK pathway activation in a dose- and time-dependent manner. Serum-starved HepG2 and SMMC-7721 cells were treated with NT157 as indicated and either stimulated with IGF-1 or not before lysis. b p-IRS1 induced by NT157 decreased with time increasing. c p-IRS1 induced by NT157 were degraded by proteasome. HepG2 cells were treated with NT157 (2  $\mu$ M) with or without MG132 (10  $\mu$ M). d NT157 inhibited phosphorylation of IGF-1R and AKT. HepG2 cells were treated with IGF-1 (50 ng/mL) with or without NT157 (2  $\mu$ M). After 24 hours, phosphorylation of the indicated proteins was examined by immunoblots analysis and quantitated by densitometry. e the ERK-MAPK pathway mediated the NT157-induced Ser-phosphorylation of IRS1/2. Serum-starved HepG2 cells were treated with PLX4720 for 30 minutes, and then NT157 and IGF-1 were added for 8 hours and lysed. f the ERK-

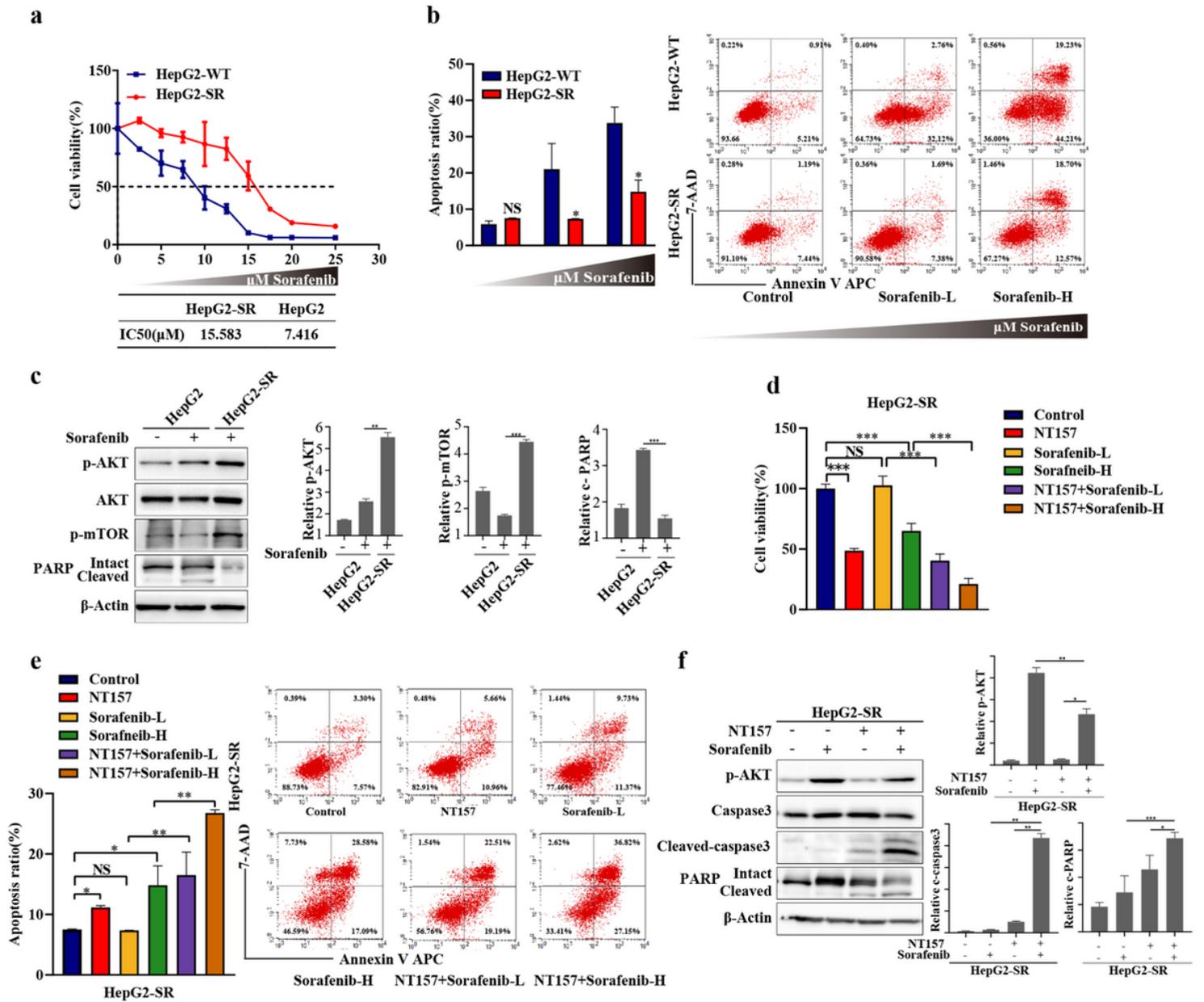
MAPK pathway mediated the antiproliferative effect of NT157. HepG2 and SMMC-7721 cells were treated with PLX4720 and NT157 for 48 hours and then survival was quantified by MTT. Experiments were performed in triplicate and repeated three times. Error bars, mean  $\pm$  SD. \*P < 0.05, \*\*P < 0.01.



**Figure 4**

NT157 and sorafenib show synergy in inducing HCC cell apoptosis. a Combination treatment with NT157 and sorafenib inhibited HCC cells proliferation. MHCC-97L, HepG2, SMMC-7721, and Huh7 cells were

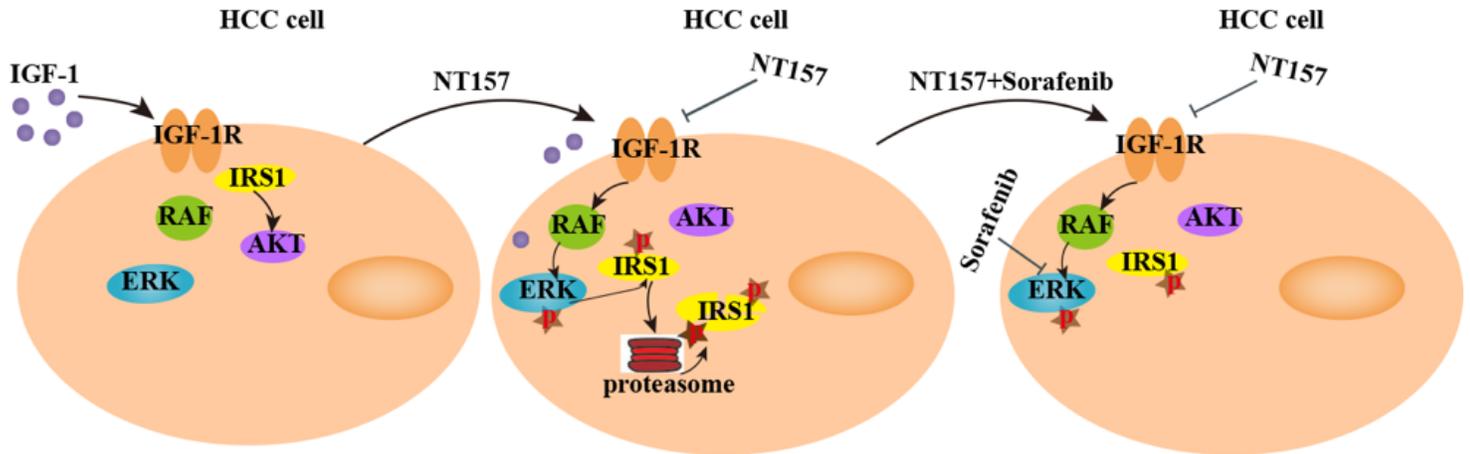
treated for 2 days with a combination of up to 3  $\mu\text{M}$  NT157 and up to 10  $\mu\text{M}$  sorafenib. Survival was assayed by MTT, and each experiment was repeated at least 3 times. Color bars indicate percentage survival normalized to untreated control cells. Representative cooperativity screens and Loewe plots for drug cooperativity are shown. b, c Combination treatment with NT157 and sorafenib induced apoptosis. Apoptosis analyses using Annexin V-APC/7-AAD double-staining (b) and immunoblots (c) with anti-caspase-3 and anti-PARP antibodies were carried out in HepG2 and SMMC-7721 cells treated with NT157 in the presence or absence of sorafenib for 24 hours. d Sorafenib attenuated NT157-activated p-ERK. HepG2 and MHCC-97L cells were treated with NT157 (2  $\mu\text{M}$ ) with or without sorafenib (5  $\mu\text{M}$ ) for 24 hours. e NT157 enhanced sorafenib's antitumor efficacy in vivo. NT157 (100 mg/kg, intraperitoneal injection) with or without sorafenib (15 mg/kg, oral gavage) was given to mice bearing HepG2 tumors for 16 days. Tumor volume was measured every 2 days after treatment; xenograft growth curves and tumor weight are shown. f NT157 had less effect on change of body weight. g Formalin-fixed, paraffin-embedded sections were stained with TUNEL and Ki67 using IHC. 200  $\times$  (Scale bar, 50  $\mu\text{m}$ ) and 400  $\times$  (Scale bar, 20  $\mu\text{m}$ ). Sorafenib-L: sorafenib-low dose (5  $\mu\text{M}$ ); Sorafenib-H: sorafenib-high dose (10  $\mu\text{M}$ ). Data are expressed as mean  $\pm$  SD and representative of 3 independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\*  $P < 0.001$ , NS: no significance.



**Figure 5**

NT157 can sensitize HCC cells to sorafenib by down-regulation of p-AKT. a-c Establishment and verification of sorafenib-resistant HepG2 cell lines. HepG2-WT: parental HepG2 cell; HepG2-SR: sorafenib-resistant HepG2 cell. (a) The cytotoxic effects of sorafenib in parental and resistant HepG2 cells. Cells were exposed to sorafenib at the indicated concentrations for 48 hours, and cell viability was analyzed by MTT assay. IC<sub>50</sub> are shown (bottom). (b) The apoptotic effect of sorafenib in parental and resistant HepG2 cells. Cells were exposed to sorafenib at the indicated concentrations for 48 hours, and the apoptotic assay used Annexin V-APC/7-AAD double-staining by flow cytometry. (c) Activation of p-AKT was associated with resistance to sorafenib in HepG2. Parental and resistant HepG2 cells were exposed to sorafenib at the indicated doses for 24 hours. Analysis of p-AKT, caspase-3, and PARP in parental and resistant HepG2 cell lines by immunoblots analysis. d-f Combination of NT157 and sorafenib induced significant cell apoptosis in resistant cells. Resistant cells were exposed to sorafenib and/or NT157 at the

indicated concentrations for 24 hours. (d) Cell viability was analyzed by MTT. (e) Apoptotic assay was analyzed by flow cytometry. (f) NT157 inhibited p-AKT and induced apoptosis in HepG2-SR cells. HepG2-SR cells were treated with sorafenib and/or NT157 for 24 hours. Cell lysates were analyzed for p-AKT, caspase-3, and PARP by immunoblots analysis. Sorafenib-L: sorafenib-low dose (5  $\mu$ M); Sorafenib-H: sorafenib-high dose (10  $\mu$ M). Experiments were performed in triplicate and repeated three times. Error bars, mean  $\pm$  SD. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, NS: no significance.



**Figure 6**

Schematic diagram. In our proposed model, NT157 phosphorylates IRS-1 through ERK-MAPK signaling to be degraded by the ubiquitin-proteasome pathway, lowers p-AKT to turn off IGF-1R signaling to inhibit proliferation and NT157 sensitizes HCC cells to sorafenib by down-regulation of p-AKT.

## Supplementary Files

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